

IN THE SPECIFICATION

Please insert the following section header on page 1, after line 8 and before line 9:

BACKGROUND OF THE INVENTION

Please insert the following paragraphs on page 5, after line 3 and before line 4:

BRIEF DESCRIPTION OF FIGURES

Figure 1: Schematic diagram of classical and alternative pathways of complement activation.  
Enzymatic components, dark grey. Anaphylatoxins enclosed in starbursts.

Figure 2: Purification of *O. moubata* complement inhibitor (OmCI). a. Cation exchange chromatography. Peak containing inhibitor indicated by arrowhead. b. Classical haemolytic assay. Sample 1 (black bar), 100% lysis; sample 2, 0% lysis; sample 3, (cross-hatched bar) serum only; sample 4, serum plus 1  $\mu$ l SGE; samples 5-23 (grey bars) serum plus 10  $\mu$ l fractions 10-28 shown in panel a. Average of 3 replicates.

Figure 3: Analysis of purified OmCI by a. denaturing SDS-PAGE, b. isoelectric focusing (IEF) and c. high pressure liquid chromatography (HPLC). Fractions f15 and f17 in panels a. and b. are the same. Fraction f15 was recovered and analysed by HPLC, panel c. Size markers and isoelectric point (PI) markers are indicated at left of panels a. and b.

Figure 4: Primary sequence of OmCI. Signal sequence underlined. Cysteine residues in bold type. Nucleotide and amino acid number indicated at right.

Figure 5: Clustal X sequence alignment of OmCI with tick salivary gland proteins 2 and 3 (TSGP2 and 3) and moubatin. Identical residues are highlighted in grey (cysteines in black) and

asterisked. The amino acid sequences are designated as follows: OmCI (SEQ ID NO: 6); TSGP3 (SEQ ID NO: 7); TSGP2 (SEQ ID NO: 8); and Moubatin (SEQ ID NO: 9).

**Figure 6:** Inhibitory activity in **a.** supernatant and **b.** cell pellet of yeast clones with OmCI inserted into genome (13.1-13.5), and clone with vector only inserted into genome (control).

**Figure 7:** Expression (**a**) and deglycosylation (**b**) of yeast cell expressed rOmCI. **a.** SDS PAGE of fractions 9-13 from Superdex-75 gel filtration column. **b.** Effect of PNGaseF treatment on mobility of highly glycosylated rOmCI (fractions 9-11 in panel **a**). Arrows indicate PNGaseF (upper arrow) and native OmCI (lower arrow). EV504 is distantly related to OmCI and is known to be glycosylated. Size markers (kDa) indicated at left of panel.

**Figure 8:** Inhibition of lysis caused by classical (CH50) and alternative (AH50) pathways of complement activation by different concentrations of native OmCI. Average of 4 replicates.

**Figure 9:** Effect of OmCI on addition of C8 and C9 to partially formed membrane attack complex (MAC). Absorbance due to 100% and 0% lysis and in absence (PBS) and presence (SGE) of inhibitor shown. Average of 6 replicates.

**Figure 10:** Timecourse showing absence of effect of OmCI on classical pathway cleavage of C3a from C3 $\alpha$  analysed by **a.** denaturing SDS-PAGE and **b.** immunoblot with C3a specific antisera **a.** Minutes (min) since start of reaction indicated. Reactions performed with (OmCI) or without (PBS) inhibitor, or in presence of 10 mM EDTA. Positions of bovine serum albumin (BSA) and haemoglobin (HAE) shown. Size markers (kDa) indicated at left of panel. **b.** As panel **a.**, positions of C3a and C3 $\alpha$  shown.

**Figure 11:** Effect of OmCI on classical, alternative and cobra venom factor (CVF) C5 convertase cleavage of C5a from C5 $\alpha$  analysed by ELISA. Picograms/ $\mu$ l C5a released measured after 100%

lysis of sheep red blood cells with water, 0% lysis in GVB<sup>2+</sup> only, and reactions with (OmCI) or without (PBS) inhibitor. Average of 4 replicates.

**Figure 12:** Effect of addition of pure C3 and C5 to C3 and C5 depleted sera on classical pathway lysis of sheep red blood cells in presence (+) and absence (-) of minimal amount of OmCI that gave complete inhibition of lysis at 1 log fold excess. Average of 4 replicates.

**Figure 13:** Effect of boiling on inhibitory activity of OmCI in CH50 assay.

**Figure 14:** Effect of pH treatment on inhibitory activity of OmCI in CH50 assay.

**Figure 15:** Detection of C5 binding to nOmCI. nOmCI and RaHBP2 (control) transferred to nitrocellulose were probed with I<sup>125</sup> labelled C3 or C5 then autoradiogramed. Protein size markers (kDa) indicated at left of panel.

**Figure 16:** Detection of nOmCI binding to C5 by gel filtration chromatography. Radiolabelled nOmCI a. with or without purified C3 and C5 (pure C3/C5) and b. with or without NHS, and C3 or C5 depleted sera (delta C3/C5). Protein size markers (kDa) indicated by arrows.

#### DETAILED DESCRIPTION OF THE INVENTION

Please amend the paragraphs at page 7, line 3 through to page 8, line 2 as follows:

Methods for the identification of homologues of the OmCI sequence given in FIG. 4 will be clear to those of skill in the art. For example, homologues may be identified by homology searching of sequence databases, both public and private. Conveniently, publicly available databases may be used, although private or commercially-available databases will be equally useful, particularly if they contain data not represented in the public databases. Primary databases are the sites of

primary nucleotide or amino acid sequence data deposit and may be publicly or commercially available. Examples of publicly-available primary databases include the GenBank database (<http://www.ncbi.nlm.nih.gov/>), the EMBL database (<http://www.ebi.ac.uk/>), the DDBJ database (<http://www.ddbj.nig.ac.jp/>), the SWISS-PROT protein database (<http://expasy.heuge.ch/>), PIR (<http://pir.georgetown.edu/>), TrEMBL (<http://www.ebi.ac.uk/>), the TIGR databases (see <http://www.tigr.org/tdb/index.html>), the NRL-3D database (<http://www.nbrfa.georgetown.edu>), the Protein Data Base (<http://www.rcsb.org/pdb>), the NRDB database (<ftp://ncbi.nlm.nih.gov/pub/nrdb/README>), the OWL database (<http://www.biochem.ucl.ac.uk/fbsm/dbbrowser/OWL/>) and the secondary databases PROSITE (<http://expasy.heuge.ch/sprot/prosite.html>), PRINTS (<http://iupab.leeds.ac.uk/bmb5dp/prints.html>), Profiles ([http://ulrec3.unil.ch/software/PFSCAN\\_form.html](http://ulrec3.unil.ch/software/PFSCAN_form.html)), Pfam (<http://www.sanger.ac.uk/software/pfam>), Identify (<http://dna.stanford.edu/identify/>) and Blocks (<http://www.blocks.fhere.org>) databases. Examples of commercially-available databases or private databases include PathoGenome (Genome Therapeutics Inc.) and PathoSeq (Incyte Pharmaceuticals Inc.).

Typically, greater than 30% identity between two polypeptides (preferably, over a specified region) is considered to be an indication of functional equivalence and thus an indication that two proteins are homologous. Preferably, proteins that are homologues have a degree of sequence identity with the OmCI protein sequence identified in FIG. 4 of greater than 60%. More preferred homologues have degrees of identity of greater than 70%, 80%, 90%, 95%, 98% or 99%, respectively with the OmCI protein sequence given in FIG. 4. Percentage identity, as referred to herein, is as determined using BLAST version 2.1.3 using the default parameters specified by the NCBI (the National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/>) [Blosum 62 matrix; gap open penalty=11 and gap extension penalty=1].

Please delete the following paragraphs from page 21, line 20 through to page 23, line 13

as follows:

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**Figure 14: Effect of pH treatment on inhibitory activity of OmCl in CH50 assay.**

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**Figure 16: Detection of nOmCl binding to C5 by gel filtration chromatography. Radiolabelled nOmCl a. with or without purified C3 and C5 (pure C3/C5) and b. with or without NHS, and C3 or C5 depleted sera (delta C3/C5). Protein size markers (kDa) indicated by arrows.**

Please amend the paragraph at page 24, line 30 through to page 25, line 14 as follows:

150  $\mu$ l SGE were diluted in 5 ml 25 mM sodium phosphate buffer pH 6.8, 50 mM NaCl and loaded onto a 1 ml Q-SEPHAROSE™ Q-Sephadex HP cation exchange column (Pharmacia) at a flow rate of 1 ml/min. After washing with a further 10 column volumes of running buffer, bound proteins were eluted using a 40 min 0.05-0.75M NaCl gradient at a flow rate of 0.5 ml/min and monitored at 280 nm. One ml fractions were collected and 10  $\mu$ l assayed for complement inhibitory activity in 200  $\mu$ l total volume CH50 assays. Representative active and inactive fractions were concentrated to 50  $\mu$ l using Centricon 3 filtration devices (Amicon), 2 ml PBS was added, the fractions were concentrated to 50  $\mu$ l again and 1.5  $\mu$ l of each was run on a 4-12% Tris-Tricine denaturing SDS gel (Invitrogen). Five  $\mu$ l per lane of both active and inactive fractions were run on a pH 3-7 IEF gel (Invitrogen) and electroblotted to IMMOBILON™-P Immobilon™-P (Millipore) using 0.7% acetic acid. The membrane was stained with Ponceau-S, and major bands excised and eluted in 200  $\mu$ l, 50 mM Tris pH 8, 2% Triton-X100 by vortexing for 1 min and centrifuging for 10 min at 15 K rpm three times. The Triton-X100 was removed by

repurifying the proteins on Q-SEPHAROSE™ Q-Sepharose HP columns using the conditions described above. After Centricon 3 concentration and buffer exchange to PBS, samples were assayed for complement inhibitory activity and examined on 4-12% gels or subjected to HPLC fractionation and protein sequence analysis.

Please amend the paragraphs at page 27, lines 10-32 as follows:

Twenty plaques from each library were picked into 0.5 ml SM buffer (0.1M NaCl, 8 mM MgSO<sub>4</sub>, 50 mM TRIS.HCl pH 7.5, 0.01% gelatin) 1% chloroform and eluted from agarose plugs by vortexing. Phage insert sizes were examined by PCR using T7 (T7 5'TAA TAC GAC TCA CTA TAG 3'; SEQ ID NO: 10) and T3 (5'AAT TAA CCC TCA CTA AAG 3'; SEQ ID NO: 11) primers. Each 100  $\mu$ l reaction comprised 2  $\mu$ l eluted phage, 2  $\mu$ l 10mM dNTPs, 2  $\mu$ l of each primer (from stocks of 0.5 $\mu$ g/ml), 10  $\mu$ l 10X REDTaq (Sigma) PCR reaction buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 11 mM MgCl<sub>2</sub>, 0.1% gelatin), 3  $\mu$ l REDTaq (Sigma) DNA polymerase (1 unit/ $\mu$ l in 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% Tween 20, 0.5% Igepal CA-630, inert dye, 50% glycerol) and 79  $\mu$ l ddH<sub>2</sub>O. Thermal cycling (Hybaid Touchdown thermal cycler) parameters were 1X 94°C 4 min, 30X 94°C 1 min, 48.5°C 45s, 72°C 90 s, and 1X 72°C 5 min. Agarose gel electrophoresis of the PCR products showed that large library inserts were  $\geq$ 1000 base pairs and small library inserts  $\leq$ 1000 base pairs.

#### *Cloning cDNA Encoding Complement Inhibitor*

The N-terminal sequences determined for the two major peaks eluting at 53 min from the HPLC were used to design a degenerate primer (OF4) for use with the T7 primer (which binds to the UniZAP XR vector), to amplify the cDNA encoding the complement inhibitor. The sequence of OF4 was 5' GTAC WSN GGN WSN GAR CCN GT 3' (where: N=A or C or G or T; R=G or A; S=G or C; and W=A or T) (SEQ ID NO: 12). The 100  $\mu$ l reaction comprised 3  $\mu$ l large or small

cDNA library, 3  $\mu$ l mM dNTPs, 2  $\mu$ l T7 and 4  $\mu$ l OF4 (from stock of 0.5 $\mu$ g/ml), 10  $\mu$ l 10X REDTaq PCR reaction buffer, 3  $\mu$ l REDTaq DNA polymerase and 75  $\mu$ l dH<sub>2</sub>O. Thermal cycling parameters were 1X 94°C 4 min, 30X 94°C 1 min, 48.5°C 45s, 72°C 90s, and 1X 72°C 5 min.

Please amend the paragraphs at page 28, line 5 through to page 29, line 19 as follows:

Conceptual translation of the largest (c.500 bp) and most intense PCR product, derived from the small cDNA library using primer OF4 with T7, revealed a significant BlastX (Altschul *et al.*, 1997) match with the C-terminal sequence of the *O. moubata* platelet aggregation inhibitor moubatin (Waxman and Connolly, 1993). The sequence extended beyond the stop codon of the cDNA encoding the peptide. A reverse primer (OR1 5' GGG AGG CTT TCT GTA TCC 3'; SEQ ID NO: 13) matching the region beyond the stop codon was used with the T3 primer (which binds to the UniZAP XR vector) to obtain the 5' end of the cDNA. The 650 bp PCR product was cloned into the pGEM<sup>®</sup>-T Easy vector (Promega) then sequenced using additional primers OR3 5'CGT CCA ATC GOT TGA AG 3' (SEQ ID NO: 14) and OF6 5' GAC TCG CAA AGT CAT CAC 3' (SEQ ID NO: 15).

#### *Sequence Analysis*

Analyses were carried out using the GCG suite of programs (Wis. Package Version 10.1, Genetics Computer Group (GCG), Madison, Wis.) and also the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (<http://expasy.heuge.ch/>). Sequences were compared with the GenBank non-redundant (NR) protein database using the BlastX program (Altschul *et al.*, 1997) and searched against the Pfam (Bateman *et al.*, 2000) and SMART (Schultz *et al.*, 2000) protein domains. Multiple sequence alignment was performed with Clustal X (Jeanmougin *et al.*, 1998).

*Yeast Expression and Purification of OmCI*

The OmCI coding region was amplified by means of the polymerase chain reaction (PCR; 95 °C. for 30", 50 °C. for 30", 72 °C. for 30"; 18 cycles), using the forward primer OM1Y (5'-ATAGAGCTAAAATGCTGGTTGGTGACC-3') (SEQ ID NO: 16) and the reverse primers OR7a (5'ACTGAGCGGCCGCCTAGTGATGGTGATGGTGAT GACCGCAGTCCTTGAGATGGGG 3' (SEQ ID NO: 17) for his-tagged products) or OR6 (5' ACTGAGCGGCCGCCTAGCAGTCCTTGAGATGGGG 3' (SEQ ID NO: 18) non-tagged product). The primers have built-in restriction sites, such that a Sac I site is added upstream of the start codon and a Not I site downstream of the stop codon. The product was ligated between the Sac I and Not I sites of the pMETα C transfer vector (Invitrogen). The plasmid - amplified in XL1-Blue cells (Stratagene) - was transformed into the *Pichia methanolica* strains pMAD16 and pMAD11, according to the instructions of the supplier (Invitrogen). Positive clones were grown in Buffered Dextrose-complex Medium BMDY, and protein expression was induced in Buffered Methanol-complex Medium. Protein expression in the supernatant and cells of 6 positive clones was assayed every 24 hours for 5 days by CH50 lytic assay.

After 96 hours incubation, 500 ml yeast cell media was centrifuged at 6370 g for 15 mins and the inhibitor precipitated from the supernatant by addition of 30% (w/v) PEG-8000 and stirring on ice for 1 hour. Following centrifugation at 23700 g for 1 hour the protein pellet was resuspended in 50 ml 25 mM sodium phosphate buffer pH 6.8, 50 mM NaCl before centrifuging at 6,000 rpm to remove insoluble material. The clarified solution was applied to a 1 ml Q-SEPHAROSE™ Q-Sephadex-HP cation exchange column and complement inhibitory activity of fractions determined as described above. Active fractions were pooled and exchanged to 300 µl PBS using Centricon 3 filtration devices (Amicon), centrifuged at 18900 g for 10 minutes then applied to a Superdex™ 75 column (Pharmacia) at a flow rate of 0.5 ml/min using 20 mM Tris pH 7.6, 200 mM NaCl as running buffer. 0.5 ml fractions were monitored at 280 nm and collected for 30 minutes. 5 µl of each fraction was assayed for inhibitory activity and active fractions exchanged to PBS before visualisation by denaturing SDS-PAGE.

Please amend the paragraph at page 30, lines 19-30 as follows:

Following cation exchange chromatography, the active fraction eluted at 0.25M NaCl (FIGS. 2a and b, arrow). The active fraction a control fraction (FIG. 3a) were electroblotted from an IEF gel (FIG. 3b) to a PVDF membrane which was stained with Ponceau-S. The major bands were excised, eluted, repurified by cation exchange chromatography and assayed for complement inhibitory activity. Denaturing SDS PAGE showed the inhibitory activity to be associated with a triplet of proteins with masses of around 19 kDa (FIG. 3a). IEF showed the inhibitory activity to be associated with a single dominant band with a pI of approximately 4.2 (FIG. 3b, upperband carryover from fraction 17). HPLC of the PVDF eluted fraction revealed four adjacent peaks (FIG. 3c). A 17 amino acid N-terminal sequence (DSESDXSGSEPVDQFA) (SEQ ID NO: 19) obtained from the largest peak (FIG. 3c, peak D) was used to design degenerate primers that generated a PCR product from *O. moubata* cDNA library which matched the N-terminal sequence.